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The splicing factors 9G8 and SRp20 transactivate splicing through different and specific enhancers

YVON CAVALOC[1]*, CYRIL F. BOURGEOIS*, LILIANE KISTER, and JAMES STÉVENIN
Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, C.U. de Strasbourg, France

ABSTRACT

The activity of the SR protein family of splicing factors in constitutive or alternative splicing requires direct interactions with the pre-mRNA substrate. Thus it is important to define the high affinity targets of the various SR species and to evaluate their ability to discriminate between defined RNA targets. We have analyzed the binding specificity of the 30-kDa SR protein 9G8, which contains a zinc knuckle in addition to the RNA binding domain (RBD). Using a SELEX approach, we demonstrate that 9G8 selects RNA sequences formed by GAC triplets, whereas a mutated zinc knuckle variant selects different RNA sequences, centered around a (A/U)C(A/U)(A/U)C motif, indicating that the zinc knuckle is involved in the RNA recognition specificity of 9G8. In contrast, SC35 selects sequences composed of pyrimidine or purine-rich motifs. Analyses of RNA–protein interactions with purified recombinant 30-kDa SR proteins or in nuclear extracts, by means of UV crosslinking and immunoprecipitation, demonstrate that 9G8, SC35, and ASF/SF2 recognize their specific RNA targets with high specificity. Interestingly, the RNA sequences selected by the mutated zinc knuckle 9G8 variant are efficiently recognized by SRp20, in agreement with the fact that the RBD of 9G8 and SRp20 are similar. Finally, we demonstrate the ability of 9G8 and of its zinc knuckle variant, or SRp20, to act as efficient splicing transactivators through their specific RNA targets. Our results provide the first evidence for cooperation between an RBD and a zinc knuckle in defining the specificity of an RNA binding domain.

Keywords: 9G8; alternative splicing; RNA-protein interactions; splicing enhancer; SR proteins

INTRODUCTION

Pre-mRNA splicing requires five small nuclear ribonucleoprotein particles (U1, U2, U4, U5, and U6 snRNPs), as well as a large number of protein factors (see Moore et al., 1993; Krämer, 1996 for review). Selection of the splice sites and branch site, and the precise pairing of the corresponding 5′ and 3′ splice sites occur via multiple RNA–RNA, RNA–protein and protein–protein interactions. A number of factors involved in the earliest steps of the spliceosome formation have been extensively studied (Krämer, 1996), in particular a family of related factors, called SR proteins (Fu, 1995; Manley & Tacke, 1996). To date, ten SR proteins have been identified in human, with or without known homologs in *Drosophila*: ASF/SF2, also known as SRp30a (Ge et al., 1991; Krainer et al., 1991), SC35, also called PR264 or SRp30b (Fu & Maniatis, 1992; Vellard et al., 1992), another SRp30 factor, 9G8 (Cavaloc et al., 1994), SRp20, also RBP1 in *Drosophila* (Kim et al., 1992; Zahler et al., 1992), SRp75 (Zahler et al., 1993b), SRp40, SRp55, and SRp30c (Screaton et al., 1995), p54 (Zhang & Wu, 1996), and finally SRp46, a recently identified SR species (Soret et al., 1998). All these factors contain at their amino terminus one or two copies of an RNA binding domain (RBD) including the conserved RNP-1 and RNP-2 submotifs (Birney et al., 1993). At their carboxy terminus, they contain a region rich in arginine (R) and serine (S) residues, with extensive repetition of R-S dipeptides (the RS domain).

Several lines of evidence indicate that the SR proteins play an important role at several stages of the splicing reaction. First, it has been shown that all SR proteins can complement a splicing-deficient S100 cytoplasmic extract, raising the possibility that these factors may be interchangeable in the splicing reaction (Fu, 1995). Secondly, SR proteins are required to stabilize

Reprint requests to: James Stévenin, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 1 Rue Laurent Fries, BP 163, 67404 Illkirch cedex, C.U. de Strasbourg, France; e-mail: stevenin@igbmc.u-strasbg.fr.
[1]Present address: UPR 41 CNRS, Faculté de Medecine 2, Avenue du Pr L. Bernard, 35043 Rennes cedex, France.
*These authors contributed equally to this work.

the binding of U1 snRNP to the 5′ splice site (Kohtz et al., 1994), and to form the early E complex (Stacknis & Reed, 1994), in agreement with the fact that the interaction of the SR proteins with the pre-mRNA is a prerequisite for the other steps of the spliceosome assembly (Fu, 1993; Zuo & Manley, 1994). Finally, SR proteins are involved in the recruitment of the U4/U6-U5 tri-snRNP to the spliceosome (Roscigno & Garcia-Blanco, 1995).

Involvement of SR proteins in alternative splicing, specifically their ability to influence in vitro the selection of alternative 5′ splice sites in a concentration-dependent manner, has been shown for both ASF/SF2 and SC35 (Ge & Manley, 1990; Krainer et al., 1990; Fu et al., 1992). However, more complete in vitro and in vivo analyses have revealed significant differences in the activity of individual SR proteins with respect to alternative splicing (Zahler et al., 1993a; Wang & Manley, 1995; Zahler & Roth, 1995). More recently, SR proteins have been implicated in the function of RNA elements identified as splicing enhancers. Such RNA motifs, which are frequently purine rich, have been characterized in mammalian exons from fibronectin (Lavigueur et al., 1993), bovine growth hormone (Sun et al., 1993; Dirksen et al., 1994), and troponin T (Xu et al., 1993; Ryan & Cooper, 1996). They have been also identified in the *doublesex* gene involved in the sex determination cascade of *Drosophila* (Lynch & Maniatis, 1995). Most of the identified elements are located downstream of introns which show tissue-specific splicing. SR proteins, notably ASF/SF2 (Sun et al., 1993; Gontarek & Derse, 1996), and/or other SR species (Lavigueur et al., 1993; Stacknis & Reed, 1994; Heinrichs & Baker, 1995; Ramchatesingh et al., 1995; Lynch & Maniatis, 1996; Gallego et al., 1997), have been implicated in the regulation of these elements. Recently, SR proteins have also been implicated in negative regulations of alternative splicing (Kanopka et al., 1996; McNally & McNally, 1996; Gallego et al., 1997).

The role of the different SR protein domains in the constitutive splicing reaction, alternative splicing, and general interactions with RNA has been analyzed by different means. The RS domain is absolutely required for constitutive splicing (Cáceres & Krainer, 1993; Zuo & Manley, 1993; Wang et al., 1996) and functions primarily in protein–protein interactions with other SR proteins or SR protein-related polypeptides such as U1-70K or U2AF35 (Wu & Maniatis, 1993; Kohtz et al., 1994; Xiao & Manley, 1997). In addition, the RS domains of ASF/SF2 and some other SR proteins appear to be functionally interchangeable in substrate commitment assays (Chandler et al., 1997) and in a cell viability system (Wang et al., 1998) and those of the major SRp30s may function more or less equivalently as splicing activators, independently of the RNA binding domain (Graveley & Maniatis, 1998). In contrast, it seems more and more clear that SR protein-specific functions rely upon their ability to interact specifically with the pre-mRNA sequences. Until recently, however, only limited details were available concerning these interactions. Using a SELEX approach, RNA targets have been identified for ASF/SF2 and SC35 (Tacke & Manley, 1995), RBP1 (Heinrichs & Baker, 1995) and more recently for SRp40 (Tacke et al., 1997) and SRp55 (Shi et al., 1997). Unfortunately, except for SRp40, which can be easily distinguished from other SR species (Tacke et al., 1997), it remains unclear whether or not individual SR proteins, in the context of a nuclear extract, still efficiently and preferentially recognize their specific target RNA sequences.

We have previously identified a SRp30 factor, called 9G8 (Cavaloc et al., 1994). In addition to containing an RBD similar to those of SRp20 and RBP1, it contains a putative zinc knuckle, located downstream, and it represents one of the first examples of putative RNA binding protein containing an RBD and a putative zinc knuckle in tandem. Here, we present a characterization of the RNA recognition specificities of the three SRp30 factors 9G8, SC35 and ASF/SF2. We focus our study on the 9G8 factor and assess the role of the zinc knuckle in defining the RNA recognition properties. Subsequently, we studied the parameters for interaction using purified recombinant SR proteins or in the context of nuclear extracts and analyzed their role as exonic splicing enhancer.

RESULTS

The 9G8 protein encodes a bona fide zinc knuckle

Like the splicing factors SLU7 (Franck & Guthrie, 1992) and SF1 (Arning et al., 1996), the 9G8 factor possesses downstream of the N-terminal RBD a domain that bears striking similarity with the CCHC zinc-knuckle motif present in nucleocapsid proteins from retroviruses. This motif appears to be essential for the specific binding of nucleocapsid proteins to retroviral genomic RNA during particle assembly (Gorelick et al., 1988). A sequence comparison of the putative zinc knuckle of 9G8 with those described in the literature demonstrates that the peptide sequence of 9G8 includes highly conserved glycine residues at positions 5 and 8 and charged residues at positions 6 and 13 (Fig. 1A). To test whether this zinc knuckle is functional, we investigated its ability to bind zinc. Recombinant mutant and wild-type 9G8 proteins, recombinant ASF and protein markers, were probed with radiolabeled zinc. In Figure 1B, lane 3, we observe that fusion protein GST-9G8 carrying a deletion of the RS domain, but retaining the putative zinc knuckle (9G8ΔRS), binds zinc efficiently. In contrast, the same protein in which the zinc knuckle has been disrupted by mutation of the two first cysteines to glycines (9G8Zn^{m}ΔRS), and the GST-ASF

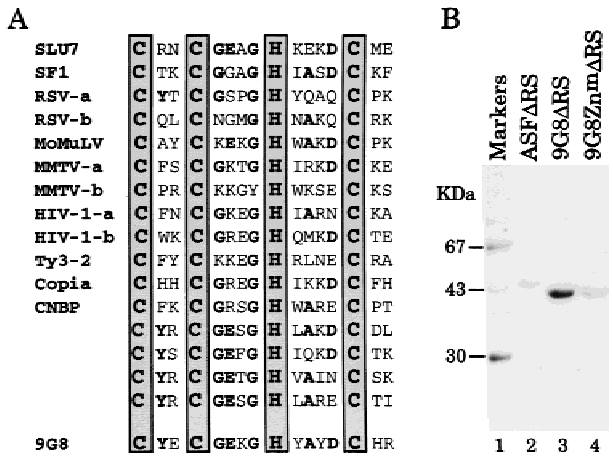

FIGURE 1. The zinc knuckle of 9G8 is functional. **A**: Amino acid sequence alignment of zinc knuckles in comparison with the 9G8 zinc knuckle. The amino acids belonging to the consensus $C(X)_2C(X)_4H(X)_4C$ are represented in larger bold type characters and are boxed. Residues that are conserved between the 9G8 zinc knuckle and the other proteins are indicated in bold type characters. Only some of the zinc knuckles of CNBP are represented in this figure. **B**: Zinc blot of different GST fusion proteins. Various GST fusion proteins (2 μg) and molecular weight markers (2 μg for each) were resolved on a 12% SDS-PAGE, transferred to nitrocellulose and probed with ^{65}Zn. The 30-kDa and 67-kDa bands revealed in the markers lane correspond to carbonic anhydrase, a zinc binding protein, and to BSA, respectively.

fusion protein, also with a deletion of its RS domain, bind zinc only poorly (Fig. 1B, lanes 4 and 2, respectively). Thus, results of Figure 1 demonstrate that 9G8 contains a genuine zinc knuckle.

9G8 and its variant mutated in the zinc knuckle recognize different RNA sequences

To identify specific RNA targets for the 9G8 protein and its derivatives, we performed an experiment based on the in vitro selection/amplification of specific ligands (SELEX; Tuerk & Gold, 1990), as described in Materials and Methods. Several precautions were taken in order to preserve specificities. (1) The GST fusion proteins used all carried deletions of the RS domain, which is thought to favor nonspecific RNA binding because of its global charge (Zuo & Manley, 1994; Xiao & Manley, 1997), as shown recently for the unphosphorylated form of SRp40 (Tacke et al., 1997). (2) The regions immediately adjacent to the RBD were preserved, as it has been demonstrated that the determinants of RNA binding specificity reside in the loops linking the structured regions of the RBD, as well as the contiguous NH_2 and COOH regions of the RBD (Burd & Dreyfuss, 1994a). After the sixth and eighth rounds of SELEX, sequence comparison of the RNA selected by 9G8 and 9G8Zn^m proteins showed significant divergence. However, selection was continued for three additional rounds to ensure that the observed divergences were not simply due to small differences in the selection efficiency. As shown in Figure 2A, one characteristic of the sequences selected by the 9G8 protein at the eleventh cycle is the occurrence of a high number of GAY triplets. Indeed, of the 56 clones sequenced, these motifs were detected 2.7 times per sequence and two consensus sequences were defined. The first consensus (AGACKACGAY), present in 45 clones, is relatively enriched for purine residues and consists mainly of a repetition of three GAC triplets or two GAC triplets flanking a UAC triplet. The second consensus (ACGAGAGAY), present in 25 clones, is more purine rich and possesses in its core a repetition of a GA doublet. In both cases, at least one GAY triplet is frequently found in the remainder of the 20-residue sequence.

The same SELEX approach was used with 9G8 carrying a mutated zinc knuckle (GST-9G8Zn^{m}ΔRS). After 11 rounds of selection, an underrepresentation of G residues was observed, and the GAY triplets that were frequently selected by the 9G8ΔRS protein were less represented (1.3 triplets per sequence). Three consensus motifs were identified from the 9G8Zn^m selected sequences (Fig. 2B). They appeared to be related, because one core motif, which consists of (A/U)C (A/U)(A/U)C sequence, is systematically present. In consensus 1, one GAY triplet follows the core motif and in consensuses 2 and 3, the core is surrounded by A, U, and C residues. Thus, these results suggest that the RBD and the zinc knuckle of the 9G8 protein act in concert to define the overall sequence specificity of this factor.

SC35 binds to various RNA sequences

With the aim of comparing the binding specificity of 9G8 with that of a second SR protein, we performed SELEX with SC35ΔRS and analyzed sequences after 8 and 11 selection cycles. The latter selection leads to an overrepresentation of one sequence (12 times in 48 clones) that did not exhibit the highest binding affinity (data not shown), and we hypothesize that it might be preferentially amplified during the selection, through a bias of the RT-PCR process. To avoid this bias, we analyzed the sequences after the ninth cycle of SELEX (Fig. 3). Of the 62 different sequences obtained, 61 fit into five consensus sequences. The two most divergent (consensus 1 and 5) are very similar to those identified by Tacke & Manley (1995). While consensus 5 (AGGAGAU) is very purine rich, consensus 1 (UGUU CSAGWU) is more balanced. In addition, we identified 3 other consensuses related to the two first: consensus 2 (GWUWCCUGCUA), consensus 3 (GGGUAUGCUG), and consensus 4 (GAGCAGUAGKS). Thus, in contrast to the 9G8 protein (this study) and to ASF/SF2 (Tacke & Manley, 1995), but in keeping with previous studies (Tacke & Manley, 1995), SC35 appears to be able to recognize different RNA sequences.

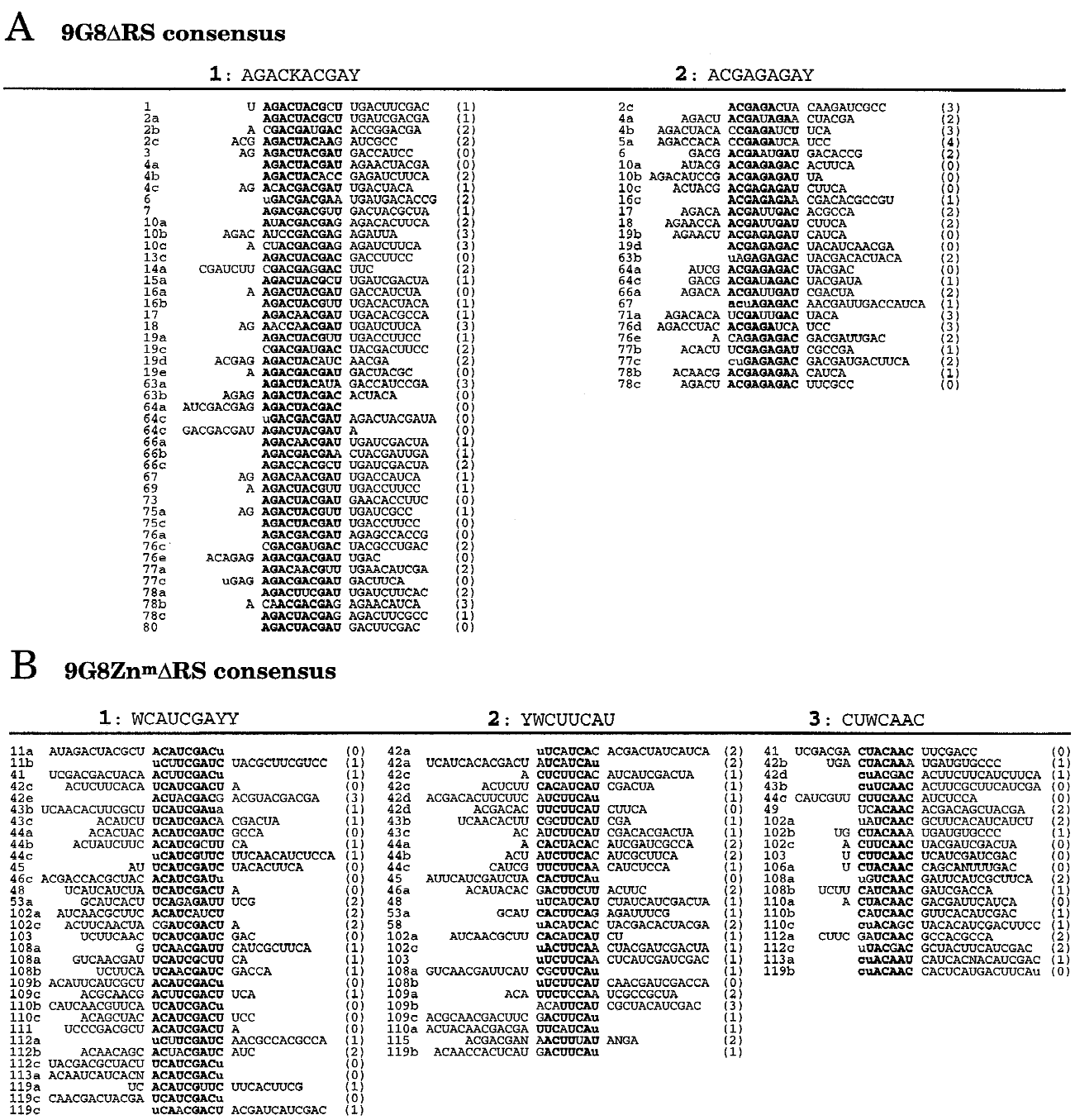

FIGURE 2. In vitro selection of 9G8ΔRS and 9G8Zn^{m}ΔRS target sequences. The consensuses identified after selection/amplification with 9G8ΔRS (**A**) or 9G8Zn^{m}ΔRS (**B**) are indicated at the top of the figure. Residues matching the consensuses are represented in bold type characters, and the mismatches are represented in standard characters. The number of mismatches is indicated in parentheses to the right of the sequences. Nucleotides shown in lower case belong to the flanking constant regions of the construction used for the SELEX experiment. Numbers to the left of the sequences identify the sequenced clones. Some sequences are listed under several consensuses. Y = C/U; K = G/U; W = A/U; S = G/C.

Interactions between recombinant SR proteins and their target sequences

RNA sequences obtained after multiple cycles of SELEX are predicted to bind efficiently to the selecting protein. To define better the binding properties of the various SR proteins, we performed gel-mobility shift experiments using an extended set of RNA sequences, identified between the sixth and eleventh SELEX cycles, which contain the different consensus sequences obtained for each SR (see Table 1). In the first quantitative analysis, we used the fusion proteins corresponding only to the three bona fide 30-kDa SR proteins (9G8, SC35, and ASF/SF2). The ASF/SF2 specific sequences

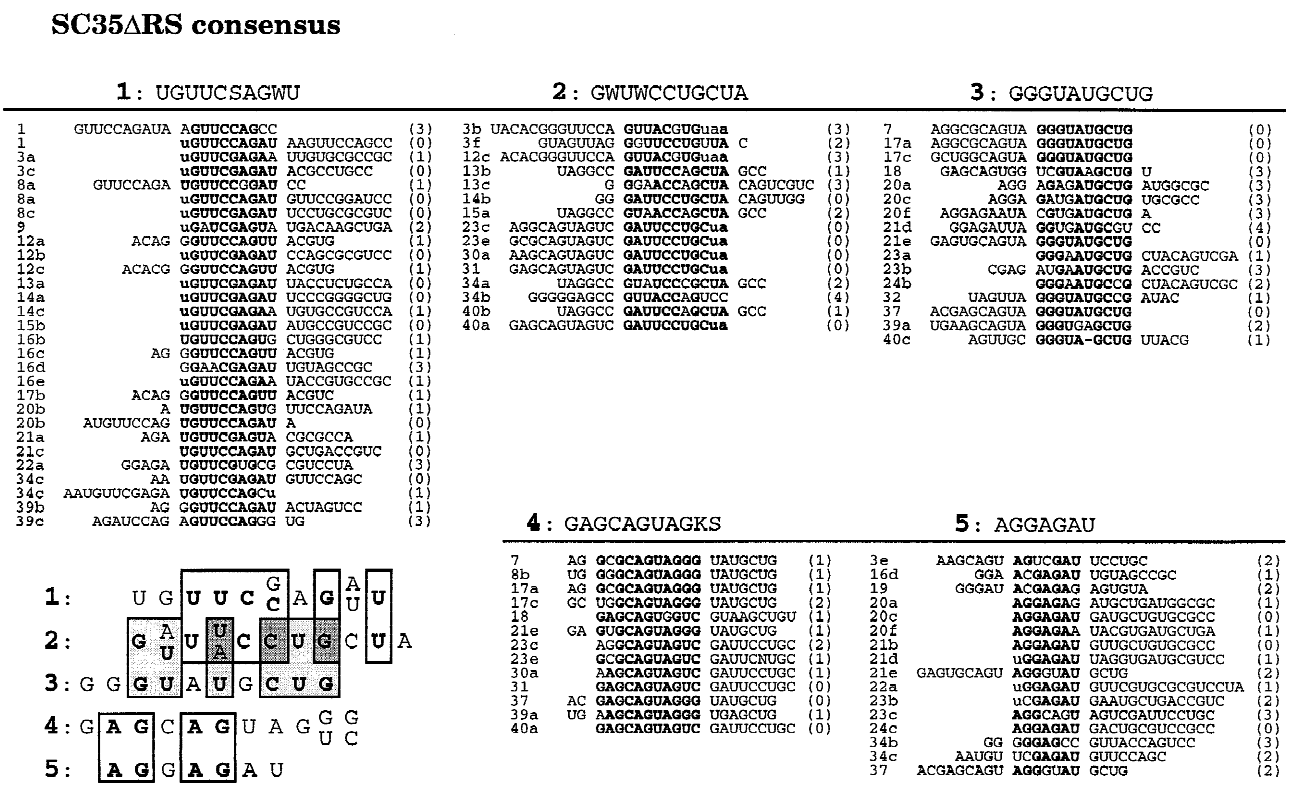

FIGURE 3. In vitro selection of SC35ΔRS target sequences. The consensuses identified after selection/amplification with SC35ΔRS are indicated at the top of each sequence panel. The representation used is the same as in Figure 2. An alignment of the five consensus sequences is represented at the bottom left of the figure. Nucleotides common to consensuses 1 and 2, 2 and 3, and 4 and 5 are in bold type characters, and are boxed, in white for consensuses 1/2 and 4/5, and in grey for consensuses 2/3. Nucleotides of consensus 2 that matched both consensuses 1 and 3 are boxed in dark grey. Note that sequence 34b, containing consensus 2, which corresponds to clone 33 isolated at the eleventh cycle, was present six times and that the sequence 12c, containing consensus 1, was present three times.

were taken from the literature (Tacke & Manley, 1995). To assess all interactions, potentially including weak interaction between nonspecific protein:RNA partners, the analysis was performed in the presence of an excess of protein (75 ng) relative to RNA.

For all 9G8-specific transcripts, we observe efficient binding (>70% or 90%) with 9G8ΔRS, and high binding specificity, as only one of the probes (78b) binds significantly with SC35 or ASF factors (Table 1). However, only 9G8 protein binds efficiently to the 78b probe in the presence of lower amounts (10–30 ng instead of 75 ng) of recombinant proteins. The SC35-specific probes that we tested contain one or two of the defined consensus sequences. All transcripts tested exhibit strong binding to SC35 (>70%), whereas none binds significantly to 9G8 and that only two (90 and S33) interact significantly with ASF, possibly because they contain the purine-rich consensus 5, which resembles the ASF-specific consensus. Finally, the ASF-specific RNA probes tested include two sequences originally tested by Tacke & Manley (1995) and one synthetic sequence (ASF-id), formed of a duplication of the GAAGAAGAAC sequence found frequently in purine-rich elements. All these RNA interact strongly with ASF (Table 1), but two of them bind to SC35, most likely because these purine-rich sequences also resemble consensus 5 of SC35, and one (ASF-id) interacts with 9G8, consistent with the regular repetition of GAA of the ASF-id probe being reminiscent of GAC repeats. Taken together, these results suggest that the interactions between the recombinant SR proteins and their respective target RNA are sequence specific.

To quantify the affinity of these interactions, we have determined the apparent equilibrium dissociation constant (K_d) of the three SR proteins for homologous transcripts (9G8-102, SC35-94, and ASF-id, respectively), using the band-shift assay, over a range of protein concentrations between 0.25 nM and 8 nM. After determination of the fraction of active protein for each fused protein by saturation RNA binding experiments (see Materials and Methods), we have obtained an apparent K_d of 0.8 ± 0.2, 0.9 ± 0.2, and 0.6 ± 0.2 nM for interactions involving recombinant 9G8, SC35, and ASF, respectively. Interestingly, these values are equivalent to those obtained for interactions between hnRNP A1 and A1-selected sequences (Burd & Dreyfuss, 1994b), as well as between Sxl protein and its wild-type se-

TABLE 1. Summary of interactions between SR proteins and selected sequences.[a]

Sequence		Selection cycle	Consensus	9G8	ASF	SC35
9G8-78b	ACAACGACGAGAGAACAUCA	11th	1 and 2	+++++	+++	+++
9G8-66c	AGACCACGCUUGAUCGACUA	11th	1	++++	+	+
9G8-102	GACAACGACGACGACUAGAA	6th	1 (duplic.)	+++++	++	++
9G8-66a	AGACAACGAUUGAUCGACUA	11th	1	+++++	+	+
9G8-13b	GGACAACAACGAUGACGACA	8th	1 (duplic.)	++++	++	+
9G8-10b	GACCAGAGAGACACAUCGAA	8th	2	++++	++	++
ASF-A10	GCACAGGACGAAGCUGCACC	7th (T&M)	2	++	++++	+
ASF-id	GAAGAAGAACGAAGAAGAAC	Chim.	1 (duplic.)	++++	+++++	++++
ASF-A14	AGGAGAACGGACAGAGCUC	7th (T&M)	1 and 2	+	+++++	+++
SC35-94	AGUGUUCCAGAUGUUCAGCC	8th	1	+	++	++++
SC35-90	GGAGGAGAACACAAGCUGAC	8th	5	+	++++	++++
SC35-S33	AAGAGAGGAGAGGUGGAG	9th (T&M)	5	+	++++	+++++
SC35-1	GUUCCAGAUAAGUUCCAGCC	9th	1 (duplic.)	+	+	++++
SC35-4	GGGAUUCCUGCUACAGUUGG	9th	2	+/−	+/−	+++++
SC35-7	AGGCGCAGUAGGGUAUGCUG	9th	3 and 4	+/−	+/−	+++++
GST-80	AGCUAACUUAUCACAUGCGU	6th		+/−	+	+/−
GST-89	AUAGCCGCGAGAGUCUCUGA	6th		+	+/−	+
GST-7	CGUUCACGGGCCACGYAUAC	6th		+/−	+	+/−

[a]Constant amount (5–10 fmol) of labeled RNA probes were incubated with 75 ng (corresponding to 1.5–1.8 pmol) of each of the GST fusion proteins deleted for their RS domain (9G8ΔRS, ASFΔRS, and SC35ΔRS), and interactions have been analyzed by gel-shift assays, as described in Materials and Methods. The cycle in which they have been selected, and the consensus to which they are linked, are indicated. Sequences ASF-A10, A14, and SC35-S33 are sequences identified by Tacke & Manley (1995). Sequence ASF-id is a chimeric sequence (see text). We verified that three nonspecific RNA sequences (GST-7, 80, and 89), which were retained on a control GST-sepharose column after the sixth cycle of SELEX selection are not bound efficiently by any of the three SR proteins. The efficiency of binding of the different GST fusion proteins has been quantified using a Fuji Bio-Imager, and is expressed in percentage of shifted RNA probe: +/−: <10%; +: 10–30%; ++: 30–50%; +++: 50–70%; ++++: 70–90%; +++++: >90%.

quence target on the *tra* pre-mRNA (Valcárcel et al., 1993; Samuels et al., 1994).

Wild-type or variant zinc knuckle recombinant 9G8 has different binding specificities

By comparing the binding properties of recombinant 9G8, its mutated variant 9G8Zn^m, and another variant deleted of the zinc knuckle (9G8ΔZn) in conditions used in Table 1, we observed more limited differences than comparing different SR proteins (data not shown). We thus analyzed interactions in the presence of lower amounts (10–30 ng) of proteins (Fig. 4). The complexes formed migrate as two bands of higher and lower mobility, which correspond most likely to binding of one (1/1 complex) and two (2/1 complex) protein molecules per RNA molecule, respectively. In addition, the 9G8Zn^m variant formed RNA:protein complexes that enter in the acrylamide gel only poorly, in contrast to those formed with 9G8 or 9G8ΔZn variant (see legend of Fig. 4). Two typical examples of interactions are shown in Figure 4A for transcripts 9G8-102 and 9G8Zn^m-45. We observed that the 9G8ΔRS protein (Fig. 4A, lanes 2–4) and the 9G8Zn^{m}ΔRS protein (Fig. 4A, lanes 15–17) bind most efficiently to their respective RNA probes. Quantification of the binding has been done for several transcripts (Fig. 4B), and indicates that mutations in the zinc knuckle of 9G8 protein severely reduce (by a factor of 2–4) the recognition efficiency of both 9G8-specific probes 102 and 13b. Conversely, the presence of the natural zinc knuckle reduces the binding efficiency with the 9G8Zn^m-specific probes 45 and 113a, but more moderately (between 40 and 50% compared with 9G8Zn^m protein). All these differences of binding are sufficient, however, to lead to selection of different RNA sequence populations by 9G8 and its Zn^m variant by SELEX approach (Figs. 2 and 3) and attest to a role for the zinc knuckle in the RNA recognition specificity.

The SR proteins discriminate between specific RNA sequences in nuclear extracts

To test whether individual 30-kDa SR proteins are able to recognize and discriminate between various RNA sequences in the context of a protein complexity closer to that occurring in vivo, we assessed binding by UV crosslinking with proteins present in a nuclear extract, as well as in an S100 fraction (Fig. 5). The 9G8 and SC35-specific transcript binding resulted in a strong band with a molecular weight of 38–40 kDa, which may correspond to a protein with an initial molecular weight of 32–35 kDa (Fig. 5, lanes 3 and 7). With the S100 fraction, no band or a band of weaker intensity was detected using the 9G8 or SC35 transcripts (Fig. 5, lanes 4 and 8, respectively). With the ASF transcript, we obtained a more complex pattern with predominant bands of 85 kDa, 54 kDa, and 38 kDa (Fig. 5, lane 10),

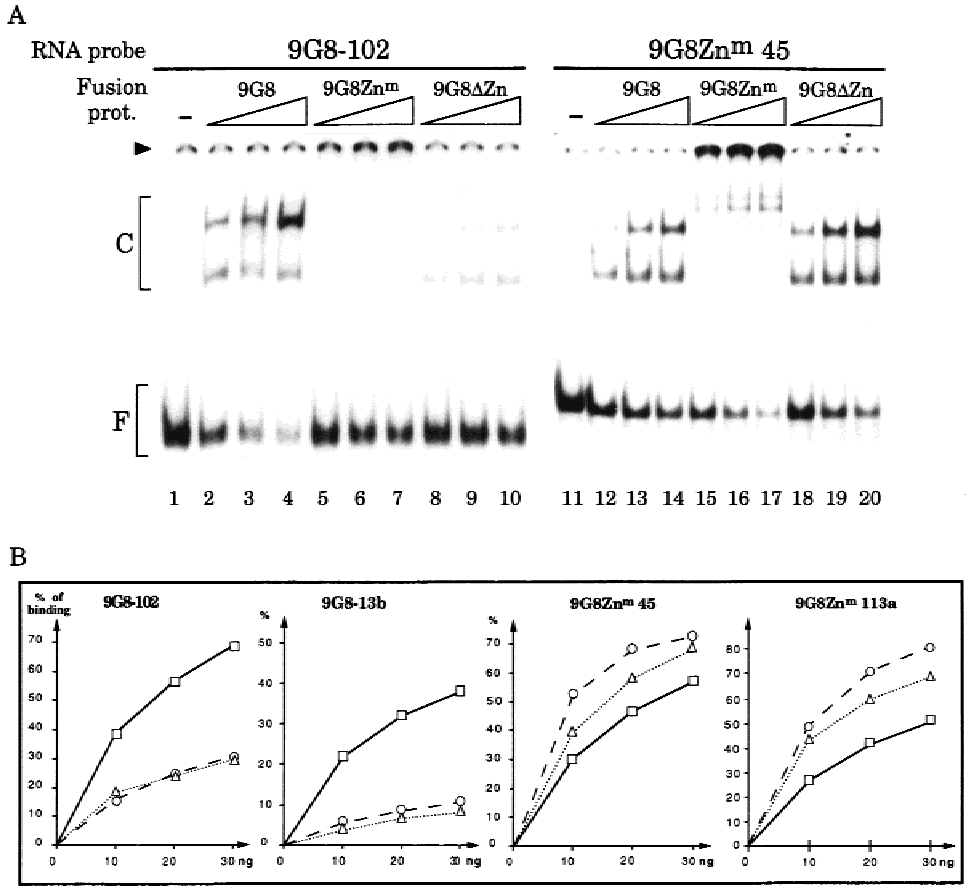

FIGURE 4. 9G8 fusion protein with natural or variant zinc knuckles have distinct binding specificities. **A**: Labeled 9G8-102 (lanes 1–10) and 9G8Zn^m 45 (lanes 11–20) RNA probes (see Table 1 for sequence), were incubated with increasing amounts (10, 20, or 30 ng) of 9G8ΔRS (lanes 2–4 and 12–14), 9G8Zn^{m}ΔRS (lanes 5–7 and 15–17) or 9G8ΔZn/RS (lanes 8–10 and 18–20), under conditions described in Materials and Methods. RNA–protein complexes (C) were resolved from free RNA probe (F) by electrophoresis on a nondenaturing polyacrylamide gel. Note that the majority of the complexes formed with the 9G8Zn^{m}ΔRS protein enter in the gel only poorly (black arrow), possibly due to the propensity of this protein to form oligomers. **B**: Quantification of binding of the three 9G8 recombinant proteins to various RNA probes. Two 9G8-specific probes (102 and 13b) and two 9G8Zn^m-specific probes (45 and 113a) have been tested, by gel-shift assays. The percentage of complexed probe, including complexes formed with 9G8Zn^m protein which stay at the start of the gel, was quantified using a Fuji Bio-Imager. Squares and full lines: 9G8ΔRS; circles and dashed lines: 9G8Zn^{m}ΔRS; triangles and dotted lines: 9G8ΔZn/RS.

but only the 38-kDa species is specific for the nuclear extract (compare Fig. 5, lanes 10 and 11). In contrast to the 9G8-specific probe, the 9G8Zn^m transcript resulted in the appearance of two strong bands of 26–27 kDa and 38 kDa, which are specific for the nuclear extract (Fig. 5, lanes 5 and 6). The same crosslinking pattern was obtained with other 9G8Zn^m probes (112a, 113a, and 42c), and with an SR population, suggesting that the 26–27-kDa band is the result of a crosslinking of the SRp20 factor to the 9G8Zn^m probe (data not shown).

To identify unambiguously the nuclear proteins that interact with the various transcripts, we performed immunoprecipitations of the UV crosslinked proteins with the specific antibodies directed against the three SRp30 proteins, because these SR species cannot be distinguished according to their molecular weight. As shown in Figure 6, we observe that the 38-kDa protein induced with the 9G8 transcript was recovered with the 9G8 antibody (Fig. 6, lane 2), but not with the SC35 or ASF antibodies (Fig. 6, lanes 3 and 4). Similarly, the SC35-specific transcript interacts only with SC35 (Fig. 6, second panel, compare lane 6 to lanes 7–8), indicating that these two specific transcripts interact efficiently and specifically with their homologous factors. The ASF-specific transcript binds efficiently to ASF/SF2 (Fig. 6, lane 10), but a weak amount of 9G8 and traces of SC35 were also revealed (Fig. 6, lanes 11–12). Because we did not have specific antibodies against SRp20, we verified that both labeled 27-kDa and 38-kDa proteins interacting with the 9G8Zn^m RNA probe are precipitated by a monoclonal antibody equivalent to mAb 104 (mAb 10H3), which recognizes the SR species, whereas the 9G8 antibody precipitated only the 38-kDa protein (data not shown). Thus, we show that the RNA targets selected by the zinc knuckle variant of 9G8 are recognized by SRp20, in agreement

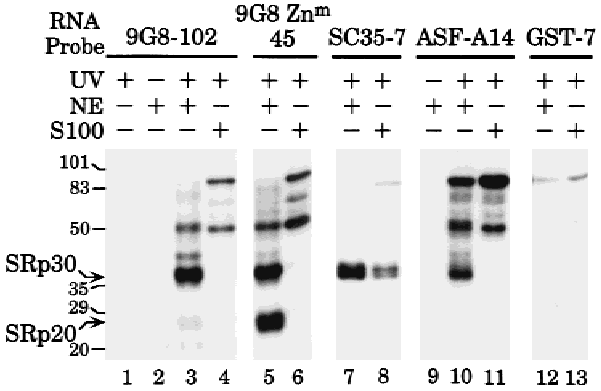

FIGURE 5. RNA targeted for SR proteins are recognized by 30- and 20-kDa nuclear proteins. Labeled RNA probes (9G8-102: lanes 1–4; 9G8Zn^m 45: lanes 5–6; SC35–7: lanes 7–8; ASF-A14: lanes 9–11, and GST-7: lanes 12–13), were incubated with 2 μL of HeLa nuclear extract or S100 cytoplasmic fraction and UV crosslinked as indicated at the top of the lanes. The proteins bound to RNA were analyzed by SDS-PAGE after RNase treatment. Controls without nuclear extract (lane 1 and data not shown) and without UV irradiation (lanes 2 and 9 and data not shown) were also performed in this experiment. Molecular weights and the position of SRp30 and SRp20 proteins are indicated on the left. Note that we have detected a low amount of SC35 in the S100 fraction.

with the fact that the RBDs of 9G8 and SRp20 are very similar (78% of identity, but only 37% with SC35 RBD or 45% with the first ASF/SF2 RBD; see Cavaloc et al., 1994). These targets are also recognized by the 9G8 factor, most likely because a part of the 9G8 population is able to interact with RNA via its RBD alone. Thus, these data demonstrate that the SR species present in nuclear extracts are able to recognize their respective SELEX-defined target RNA sequences in a very specific manner.

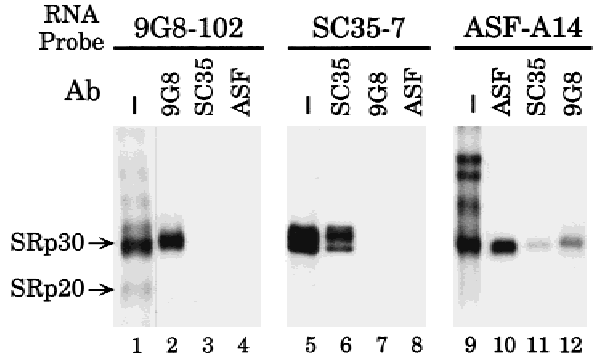

FIGURE 6. Endogenous 9G8, SC35, and ASF/SF2 discriminate between their specific target RNA. 9G8-102- (lanes 1–4), SC35–7- (lanes 5–8), and ASF-A14- (lanes 9–12) labeled RNA probes were UV crosslinked to proteins of nuclear extract, treated by RNAse, and a small aliquot was analyzed directly as in Figure 5 (lanes 1, 5, and 9). Each assay was then divided into three parts that were immunoprecipitated with antibodies (Ab) directed against 9G8 (lanes 2, 7, and 12), SC35 (lanes 3, 6, and 11), or ASF (lanes 4, 8, and 10), pre-bound to protein A (anti-ASF) or protein G (anti-9G8 or SC35) sepharose. The positions of SRp30 and SRp20 proteins are indicated on the left of the figure. The amount of immunoprecipitated sample loaded corresponds to fourfold the amount of samples directly analyzed in lanes 1, 5, and 9.

High affinity SR binding sites function as splicing enhancers in vitro

To assess whether the sequences that we selected for the various SR proteins have a functional significance, we tested their ability to replace the natural, purine-rich splicing enhancer present in the ED1 exon of the fibronectin gene (Lavigueur et al., 1993). The substrate that we tested includes the fibronectin ED1 exon flanked by its intronic sequences (Fig. 7A). Each sequence of 20 bp was inserted as a single copy in the same context as in the SELEX experiment. Results of in vitro

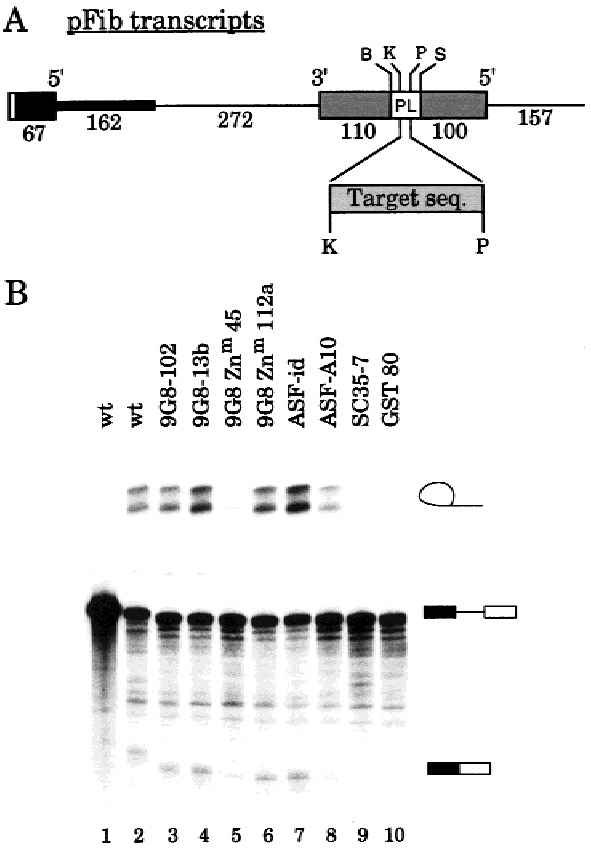

FIGURE 7. 9G8- and 9G8Zn^m-specific RNA targets can act as splicing enhancers in vitro. **A**: Schematic representation of the substrate used in in vitro splicing assays. 5′ and 3′ splice sites are indicated, and numbers below the transcript represent the length of the corresponding exonic and intronic regions. Adenovirus-derived sequences (the first exon and the beginning of the intron) are represented in black, and fibronectin sequences are in dark grey. SR-specific RNA targets (light grey; for their precise sequence, see Table 1) were inserted in a polylinker located in the EDA exon of fibronectin gene. Restriction sites: B: *Bst*EII; K: *Kpn*I; P: *Pst*I; S: *Stu*I. **B**: In vitro splicing assays. The different substrates were incubated 1.5 h with nuclear extract (lanes 2 to 10) and analyzed under standard conditions. Lane 1: wild-type transcript without nuclear extract. Pre-mRNA and products of the splicing reaction (intron and mRNA) are schematized at the right of the figure.

splicing are shown in Figure 7B. We observe that the reference transcript, which contains the wild-type purine-rich enhancer, is significantly spliced (Fig. 7B, lane 2), whereas the insertion of the control GST-80 sequence, which does not interact with SR proteins, does not promote the splicing activity (Fig. 7B, lane 10). In contrast, insertion of 9G8- and ASF/SF2-specific sequences results in a significant restoration of splicing (Fig. 7B, lanes 3 and 8) or in a stronger activation than with the natural enhancer of splicing (Fig. 7B, lanes 4 and 7). Results are less clearcut with 9G8Zn^m-specific sequences, as a strong stimulation of splicing is induced by the sequence 112a (Fig. 7B, lane 6), while sequences 45 (Fig. 7B, lane 5) and 113a (data not shown) have a lower enhancer activity. We verified that the differences in enhancer activity between sequences 112a, 45, and 113a are not directly related to an imbalance of their relative binding efficiencies to the two proteins SRp20 and 9G8 (data not shown). Finally, although the SC35-7 sequence binds SC35 factor very efficiently (Fig. 6), no activation of splicing was observed with this sequence (Fig. 7B, lane 9). Four other SC35-specific sequences (sequences 94, 90, 1, and S33, depicted in Table 1) also did not enhance splicing (data not shown). The results of Figure 7 have been obtained with another model of alternative splicing, based on the K-SAM exon of the FGFR2 gene, whose alternative splicing is related in part to a weak 3′ splice site (Del Gatto & Breathnach, 1995; data not shown), confirming that 9G8, 9G8Zn^m, and ASF/SF2 high affinity targets may serve as efficient splicing enhancers, which was not the case for the SC35-specific targets.

SR species preferentially activate splicing through interaction with their specific targets

To determine whether each SR species activates splicing via its specific high affinity target, we used the experimental approach defined previously by Tacke & Manley (1995), in which they showed that a splicing-deficient S100 extract has to be supplemented with a 20–40% ammonium sulfate fraction of nuclear extract (NF 20–40) to promote splicing of introns with weak splicing signals. When we used this combination with the fibronectin transcripts tested in Figure 7, only a poor splicing activation was obtained in the presence of total or individual SR species (data not shown), most probably because the initial substrate is already difficult to splice in the presence of nuclear extract (see Fig. 7, lane 2). To overcome this problem, we defined a more appropriate splicing substrate based on the adenoviral E1A pre-mRNA. Indeed, as shown in Figure 8B, the Sp1 transcript, which contains the wild-type sequence of the E1A unit (Schmitt et al., 1987), is highly spliced by a standard nuclear extract (lane 1), but is not spliced when the exon 2 sequence is in the antisense orientation (lane 2), suggesting that some *cis*-activators are required to reinforce the weak 3′ splice site U_6AAAAG/G. Interestingly, the insertion of a single copy of the high affinity targets for 9G8, 9G8Zn^m, and ASF/SF2, 50 nt downstream of the 3′ splice site in the inverted exon 2, results in a very strong splicing activation (Fig. 8B, lanes 3–8). These activations are sequence specific because the insertion of the SC35-specific targets results only in no or poor splicing (Fig. 8B, lanes 9 and 10), as shown also for the nonrelated GST-80 sequence (Fig. 8B, lane 11). Thus, the activation pattern obtained with the E1A constructs is very similar to that observed previously in Figure 7, except that the splicing activations are stronger. In fact, with the particularly highly active nuclear extract tested in Figure 8C, we even note that the SC35 enhancer-containing transcript was significantly spliced (Fig. 8C, lane 21), although less efficiently than the other tested transcripts (Fig. 8C, lanes 1, 8, and 15).

Splicing activation in assays containing the combination of S100, the NF20–40 fraction, and E1A transcripts containing specific enhancers was performed in the presence of an equivalent amount of each SR protein (see the legend of Fig. 8C). A comparison of 9G8 and 9G8Zn^m enhancer-containing transcripts shows that 9G8 protein promotes significant splicing activation of the 9G8-enhancer transcript (Fig. 8C, lane 3), whereas it reacts only weakly with the 9G8Zn^m enhancer transcript (Fig. 8C, lane 12). In contrast, the 9G8Zn^m and SRp20 proteins activate efficient splicing of the 9G8Zn^m-specific transcript (Fig. 8C, lanes 10–11), whereas they were poorly efficient (Fig. 8C, lanes 4–5) at splicing the 9G8-specific transcript. Both heterologous SR species (ASF/SF2 and SC35) for the 9G8 or 9G8Zn^m transcripts only weakly activate splicing (Fig. 8C, lanes 6–7 and 13–14). Interestingly, these data indicated that: (1) the constitutive splicing activity of the zinc-knuckle-mutated variant of the 9G8 protein is preserved, as well as its activity as a transactivator; (2) 9G8 on one hand and SRp20 and 9G8Zn^m protein on the other hand possess distinct and specific capacities to activate enhancer-dependent splicing reactions.

Finally, comparable assays have been performed with the ASF/SF2 and SC35 specific transcripts. With the first, the best activation of splicing was obtained by addition of ASF/SF2 (Fig. 8C, lane 17). The addition of 9G8 and SC35 also led to significant activation (Fig. 8C, lanes 18 and 20), consistent with the fact that both these SR proteins are able also to interact with the ASF-specific target (see Fig. 6), possibly through the involvement of Tra2 (Beil et al., 1997) present in the NF20–40, because it has been shown to improve splicing dependent of ASF/SF2 specific enhancers (Tacke et al., 1998). In contrast, the SC35-specific transcripts 7 (Fig. 8C, lane 23) or 94 (not shown) are only very weakly activated by the addition of SC35, in agreement with the weak activation of these transcripts observed in the presence of standard nuclear extract (Fig. 8B,

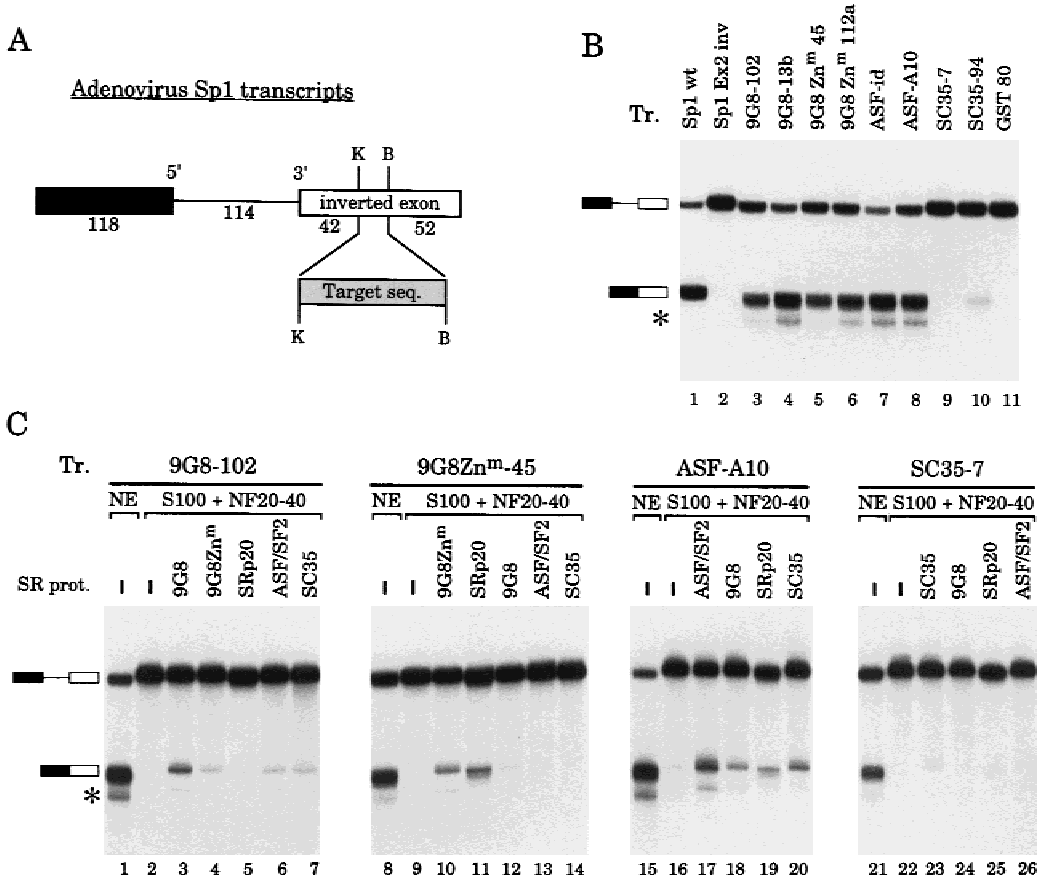

FIGURE 8. 9G8, its Zn^m variant, and SRp20 can activate enhancer-dependent splicing reactions specifically. **A**: Schematic representation of the Sp1 transcript used in in vitro splicing assays. SR-specific RNA targets (light grey) were inserted between *Kpn*I (K) and *Bam*HI (B) sites, created in the exon 2. Numbers below the transcript represent the length of the corresponding exonic and intronic regions. **B**: Standard in vitro splicing assays. Natural Sp1 (lane 1) and Sp1-derived transcripts containing no inserted sequence (lane 2), SR-specific sequences (lanes 3–10), or control GST sequence (lane 11) were spliced in the presence of standard nuclear extract and analyzed as in Figure 7. Asterisk represents a minor mRNA formed using a cryptic 3′ splice site within the inverted exon 2, located 22 nt downstream of the natural 3′ site. **C**: Activation of enhancer-dependent splicing substrates by SR proteins. Transcripts were incubated in a mixture of cytoplasmic S100 fraction and NF20–40 fraction. Assays were supplemented with no SR protein (lanes 2, 9, 16, and 22), or with individual SR proteins: 400 ng of baculovirus-purified 9G8 (lanes 3, 12, 18, and 24), ASF/SF2 (lanes 6, 13, 17, and 26) or SC35 (lanes 7, 14, 20, and 23); 800 ng of baculovirus-purified $9G8Zn^m$ (lanes 4 and 10); and 800 ng of partially purified SRp20 (lanes 5, 11, 19, and 25). Each SR protein amount was normalized to give the same level of 13S mRNA splicing in complementation assays using wild-type E1A transcript. Lanes 1, 8, 15, and 21 represent control splicing assays using nuclear extract.

lanes 9–10). The reason why SC35 appears to be poorly efficient in this process remains to be elucidated, but could be due to the absence of splicing coactivators specific for SC35 targets.

DISCUSSION

Dual RNA recognition specificity of 9G8 protein via its zinc knuckle domain

The 9G8 SR protein is the only SR species discovered to date containing a functional zinc knuckle, located between the RBD and the RS domain. In this report, we analyze the capacities of 9G8 to intervene in alternative splicing and we determine the role of the RBD and the zinc knuckle in the recognition of specific RNA targets. By using a SELEX approach, we have identified the high affinity RNA targets of the 9G8 protein, a variant of 9G8 mutated in the zinc knuckle, as well as SC35. Individual sequences that we obtained do not display any systematic secondary structure, in contrast to what was obtained for B52 or SRp40 specific sequences (Shi et al., 1997; Tacke et al., 1997).

Using recombinant 9G8, we identified a major decamer consensus based on a repetition of GAC triplets and an octamer consensus of the form AGAGAGAC. We showed that these 9G8-selected sequences are recognized only by 9G8 factor in a nuclear extract, demonstrating that they are highly specific for this factor. The RNA targets identified for the $9G8Zn^m$ re-

combinant factor revealed three consensuses, all constructed around a (A/U)C(A/U)(A/U)C pentamer, which are clearly different from the 9G8-specific consensuses. Interestingly, the 9G8Zn^m-specific binding motifs that we tested subsequently with total SR proteins and nuclear extracts are strongly recognized by SRp20, most likely because the RBDs of 9G8 and SRp20, as well as its *Drosophila* homolog RBP1, are closely related. Taken together, our results provided the first evidence for cooperation between an RBD and a zinc knuckle in forming a functional RNA binding domain. Although zinc knuckles are usually considered as nonspecific nucleic acid binding motifs, the HIV nucleocapsid protein, which contains two zinc knuckles, is able to select high-affinity targets formed from stretches of G and U residues (Berglund et al., 1997), indicating the ability of a zinc knuckle to exhibit significant recognition specificity in the appropriate environment.

To correlate directly the results of the interactions of the high affinity targets with those regarding their enhancer abilities, we have assayed three synthetic splicing substrates including a weak 3′ splice site, in which one copy of the selected target sequences was inserted, in the same sequence context as that used for the interaction experiments. Our analysis of the enhancer abilities was significantly different from those performed previously, in which three copies of the ASF/SF2- or SRp40-specific targets were inserted in a splicing substrate with a weak 5′ splice site (Tacke & Manley, 1995; Tacke et al., 1997). We have shown that a single copy of the 9G8-, 9G8Zn^m-, and ASF/SF2-specific RNA target was sufficient to obtain activation of the model substrates in splicing assays with nuclear extract (Figs. 7 and 8B). More importantly, by using complementation splicing assays, we demonstrate that the 9G8 protein and its Zn^m variant or SRp20 specifically restore splicing through an activation of the 9G8 and 9G8Zn^m enhancers, respectively (Fig. 8C). Thus, these results indicated that 9G8 and SRp20 display specific capacities as transactivators of alternative splicing, as previously shown for ASF/SF2 and SRp40 (Tacke & Manley, 1995; Tacke et al., 1997), and that the zinc knuckle is involved in the 9G8 transactivation specificity. Putative 9G8 targets, containing repeats of GAC triplets, are present in exon 5 of the cTNT gene (Elrick et al., 1998) and in the HIV *tat* exon 1 (Tacke & Manley, 1995). Such sequence is also located upstream of the 12S donor site of adenoviral E1A pre-mRNA that activates the 12S mRNA reaction specifically in the presence of 9G8 (C.F. Bourgeois & J. Stévenin, unpubl.).

That SRp20 plays a role in vivo in transactivation is also suggested by recent results showing that SRp20 is involved in the alternative splicing of the exon 4 of its own pre-mRNA (Jumaa & Nielsen, 1997). Interestingly, we identify eight repetitions of the (A/U)C(A/U)(A/U)C motif in this exon, which are identical to the core motif that we have selected with the Zn^m variant of 9G8 factor. Furthermore, by site-specific UV crosslinking, Lou and coworkers (1998) have recently shown that SRp20 interacts with a C/U-rich sequence to regulate alternative polyadenylation of calcitonin/CGRP gene. It is attractive to speculate that all these sequences rich in CA/U residues, as well as some splicing enhancers rich in C/A residues (Coulter et al., 1997), may represent ideal binding sites for SRp20. In addition, our data demonstrate that the Zn^m variant of 9G8 still exhibits a constitutive splicing activity as well as a transactivatory activity and that it is functionally equivalent to SRp20.

Finally, in addition to their recognition by SRp20, we have shown that the 9G8Zn^m-specific probes are also recognized by 9G8, as checked by immunoprecipitation. Therefore, it is reasonable to hypothesize that 9G8 might recognize RNA in two ways, either through its RBD and zinc knuckle, resulting in primary interactions with 9G8-specific targets, or through its RBD alone, leading to recognition of sequences equivalent to SRp20-specific targets. One possible explanation for the dual behavior of the 9G8 factor is that the zinc knuckle, which is very close to the RS domain and smaller than the RBD, may be masked in a fraction of 9G8 molecules, as the consequence of interactions of its RS domain with other protein factors. Interestingly, the analysis of the regulatory complex containing Tra, Tra2, and SR protein(s), which assembles on the *dsx*RE of *Drosophila*, leads to results that might be explained by such a mechanism (Lynch & Maniatis, 1996). Indeed, using an heterologous system formed from a HeLa cell nuclear extract, these authors have shown that 9G8, but not SRp20, the structural homolog of RBP1, interacts with the *dsx*RE in the presence of Tra/Tra2. Strikingly, we observe that our consensuses 2 and 3 for the 9G8Zn^m variant (YWCUUCAU and CUW CAAC) resemble one form of consensus identified by SELEX for RBP1 (ACAWCUUUA) (Heinrichs & Baker, 1995), as well as motifs of the *Drosophila dsx*RE, which have been shown to be targets for RBP1 in the presence of Tra/Tra2 using *Drosophila* nuclear extracts (Lynch & Maniatis, 1996). Moreover, six of the 9G8Zn^m-specific sequences (11b, 45, 103, 108b, 112a, and 113a) match 11 or 12 residues of the 13-nt *dsx* repeats. The molecular basis of the involvement of 9G8 in this process, which requires its cooperative interaction with Tra/Tra2, might lead to a masking of the zinc knuckle, thus rendering it functionally equivalent to RBP1. Remarkably, 9G8 has been crosslinked to the 5′ sequence UCAACA of repeat 5 of *dsx*RE (Lynch & Maniatis, 1996), which matches perfectly with consensus 3 of the 9G8Zn^m variant.

SC35 is not able to transactivate splicing through its specific RNA targets

Compared with the consensuses obtained with 9G8, 9G8Zn^m (this study), and ASF/SF2 (Tacke & Manley,

1995), results obtained with SC35 are more unexpected. We identified a panel of five different consensuses that are partially related (see Fig. 3), the two most divergent consensuses being comparable to those identified previously (Tacke & Manley, 1995). However, all tested SC35 binding sequences containing one or two copies of these consensuses are efficiently recognized by the recombinant SC35 or endogenous SC35 in the nuclear extract (Figs. 5 and 6), thus validating our SELEX selection and indicating that the identified consensuses are all bona fide SC35-specific targets.

In contrast to results obtained with other SRp30-specific targets, we have shown that the SC35-specific targets are unable to activate efficient *in cis* splicing within the two first model substrates tested, although two of five activate moderately splicing of the E1A-derived substrate in highly favorable splicing conditions (Fig. 8C). Furthermore, duplication of a SC35-specific target in the last model substrate did not result in splicing activation (data not shown). It seems unlikely that the absence or weakness of splicing activation results from a problem of accessibility of the SC35 sequences because they were inserted in the substrates in the same context as the other specific targets. Thus, we conclude that SC35 cannot activate splicing of a substrate containing a weak 3′ splice site and a single or double high-affinity target as efficiently as other SR proteins, in agreement with previous results from Tacke & Manley (1995). In fact, no clear example of natural enhancer activation by SC35 has been reported, whereas several examples of activation by ASF/SF2 have been described (Sun et al., 1993; Ramchatesingh et al., 1995; Gontarek & Derse, 1996; Lynch & Maniatis, 1996; Gallego et al., 1997). Moreover, SC35 even exhibits an antagonistic effect on ASF/SF2-activated splicing of the β-tropomyosin exon 6A, which depends on an intronic enhancer (Gallego et al., 1997). These results do not exclude, however, that SC35 might play an active role in transactivation, through cooperation with other SR proteins or splicing coactivators.

Clearly, the in vitro approach including the selection for high-affinity targets for one SR species (the "binding SELEX") followed by the assessment of these targets as specific splicing enhancers leads to the identification of well-defined consensus for ASF/SF2 (Tacke & Manley, 1995), SRp40 (Tacke et al., 1997), as well as 9G8, 9G8Zn^m, and SRp20, but not SC35 (this study). Another approach has been described recently by Liu et al. (1998) for identifying functional splicing enhancers for ASF/SF2, SRp40, and SRp55 and has been defined as "functional SELEX" by these authors. However, employing this technique with ASF/SF2 and SRp40 resulted in more degenerate RNA sequences, which were different from the sequences previously identified (Tacke & Manley, 1995; Tacke et al., 1997). This might be explained, at least in part, by the fact that only three cycles of selection were performed and that many different splicing factors may intervene during the selection, in addition to the specific SR protein being tested. The complexity of the functional SELEX is well exemplified also by the fact that it was not possible to inactivate the function of selected sequences by mutations in or around the restricted consensus motif (Liu et al., 1998). Thus, both binding and functional SELEX should be considered as complementary rather than contradictory approaches, and it could be interesting to test the functional SELEX with the SC35 factor.

Some implications for regulation of alternative splicing

Taken as a whole, the target consensuses obtained for ASF/SF2, SC35, SRp40, and 9G8 (Tacke & Manley, 1995; Tacke et al., 1997; this study) and those recognized by RBP1 (Heinrichs & Baker, 1995), SRp20 (this study), or SRp55 (Shi et al., 1997) form a set of significantly divergent sequences, extending from purine-rich sequences (ASF, SC35, and 9G8) to pyrimidine-rich sequences (SC35, RBP1, and SRp20). This is an interesting feature because it suggests a mechanism for the regulation of alternative splicing by SR proteins through the use of a large variety of RNA motifs, and induction of activation or inhibition (Kanopka et al., 1996; McNally & McNally, 1996) according their position within the pre-mRNA substrate.

The purine-rich sequences, especially in the form of GAA triplet repeats, have been the most frequently identified (for a compilation, see Tacke & Manley, 1995), and they may represent general splicing elements not necessarily involved in developmental stage-specific splicing. Clearly, all these targets are prototypical sequences and it is likely that other sequences with higher complexity and/or weaker affinity are involved in more refined splicing regulation. The best-characterized example of this is the *Drosophila doublesex* enhancer, which contains six repeats of *dsx* motifs as well as a purine-rich motif (Lynch & Maniatis, 1995). Moreover, the intronic pyrimidine-rich enhancer located downstream of the 6A exon of β-tropomyosin, which requires ASF/SF2 factor for its activation, is a good example of a weak affinity target (Gallego et al., 1997). In most of these examples, it is possible that non-SR proteins or SR protein-related polypeptides might act in cooperation with SR proteins in splicing regulation, as occurs in *Drosophila* for the splicing of *dsx* pre-mRNA owing to the involvement of the Tra/Tra2 proteins. In this respect, Tra2 might be more widely involved, because in *Drosophila* it also regulates the alternative splicing of the *fruitless* pre-mRNA (Heinrichs et al., 1998). Furthermore, the human Tra2α and β proteins possess the ability to stimulate splicing dependent on ASF/SF2 specific targets (Tacke et al., 1998). An emerging hypothesis is that the SR protein-like polypeptides, which

are able to develop interactions with SR proteins by means of their SR dipeptide repeats, could represent a family of coactivators that cooperate with SR proteins for the splicing regulation (Blencowe et al., 1998). In mammals, Tra2, as well as the homolog of SWAP (Sarkissian et al., 1996), might represent the prototype of coactivators, but other SR protein-like polypeptides, like Sip1, for SC35-interacting protein 1 (Zhang & Wu, 1998), or Urp for U2AF35-related protein (Tronchère et al., 1997), may also represent putative candidates that could be involved in splicing regulation.

MATERIALS AND METHODS

Construction and expression of SR mutants

For the construction of pGEX-9G8ΔRS, a PCR-generated DNA fragment containing the full-length coding sequence for the 9G8 protein flanked by *Nhe*I restriction sites was inserted in phase in the pGEX-3X vector. The coding sequence of the RS domain was deleted by removal of a *Nae*I/*Xho*I fragment. Then, the coding sequence for the last 14 amino acids and a five histidine tail was reconstituted by synthetic oligonucleotides (final deletion of amino acids 125–223). To produce the pGEX-9G8Zn^{m}ΔRS vector, a *Sph*I/*Nae*I fragment was deleted from pGEX-9G8ΔRS and the complete sequence was reconstituted with synthetic oligonucleotides, so that the two first cysteine residues of the zinc knuckle were mutated into two glycines. To obtain pGEX-9G8ΔZn/RS, the sequence separating the *Sph*I site from the beginning of the coding sequence of the zinc knuckle was reconstituted in the same way (final deletion of amino acids 106–223).

pGEX-ASF vector was generated by insertion of an *Eco*RI/*Hin*dIII fragment from the pDS56-ASF vector (Ge et al., 1991) into pBluescript II SK(+) (Stratagene), followed by the transfer of a *Bam*HI/*Sal*I fragment into pGEX-4T3 (Pharmacia). To yield pGEX-ASFΔRS, the coding sequence for the RS domain of ASF was removed from the pDS56-ASF plasmid by a deletion of an *Apa*I-*Hin*dIII fragment, and the sequence for the C-terminal 14 amino acids was recreated by the insertion of synthetic oligonucleotides (final deletion of the RS domain between amino acids 198 and 230) to yield pDS56-ASFΔRS. A *Kpn*I-*Hin*dIII fragment from this plasmid was substituted for the corresponding fragment of the pGEX-ASF plasmid to generate pGEX-ASFΔRS.

The coding sequence for the human SC35 protein (amino acids 1–123) was isolated as a *Not*I-*Avr*II fragment from pAcNTHisB-HPR5. The coding sequence for the C-terminal 15 amino acids and a histidine tail, flanked by *Avr*II and *Not*I restriction sites, was created by oligonucleotides and ligated to the above fragment. The resulting *Not*I fragment was inserted in frame to pGEX-4T3 (Pharmacia). The glutathione-S-transferase (GST) fusion proteins of the SR proteins were expressed in the *Escherichia coli*, BL21 strain, at 28 °C during 3 h following induction with 0.1 mM IPTG. Following the bacterial lysis, soluble proteins were affinity purified under nondenaturing conditions, on a glutathione-Sepharose 4B-CL resin (Pharmacia) according to the protocol described by the manufacturer.

Zinc blot

The Zinc blot analysis was performed as described previously (Mazen et al., 1988). After migration of the proteins on a 12% SDS-PAGE, the gel was washed in the electrophoresis buffer (25 mM Tris-Base, 192 mM Glycine, 0.1% SDS) supplemented with 0.7 M β-mercaptoethanol during 30 min at 4 °C and 1 h at 37 °C, then the proteins were electrotransferred onto a nitrocellulose membrane. After renaturation of the proteins, the membrane was incubated 45 min at 20 °C in 10 mL of a binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl), containing 2 μL of [^{65}Zn]Cl_2 (2.2 mCi/mL), washed in the same buffer without radioactive zinc (3 $\times$ 5 min) and autoradiographed.

SELEX

Two oligonucleotides, 5′-GCGTCTCTGCAGTAGTTA(N_{20})AGTCGGCATCTTGGTACCCTATAGTGAGTCGTATTACC-3′ (SA251), corresponding to the template strand (where N_{20} indicates a 20-bases random sequence) and 5′-GGTAATACGACTCACTATAGGGTACCAAGATGCCGACT-3′ (SA252), were hybridized. The resulting matrix was in vitro transcribed in the presence of 50 U of T7 RNA polymerase (Promega) as described by the manufacturer for the synthesis of large amounts of RNA. The resulting RNA population was precipitated and dissolved in the binding buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 0.1% Triton-X100, 1 mM DTT). In parallel, 1 μg of each highly purified GST fusion protein was bound to 15 μL of Glutathione-Sepharose beads and equilibrated in the binding buffer. The binding reaction was performed in the presence of 10 μg of in vitro transcribed RNA, 50 μg of *E. coli* tRNA and 35 U of RNAsin during 45 min at 20 °C. The beads were then washed extensively in 250 mM KCl and the selected RNA was released by proteolysis for 30 min at 37 °C in the presence of 20 μg of proteinase K. The eluted RNA was precipitated and resuspended in 20 μL of NPES (40 mM PIPES, pH 6.4, 250 mM NaCl, 5 mM EDTA and 0.2% SDS), hybridized to 200 ng of the oligonucleotide SA253 (5′-GCGTCTCTGCAGTAGTTA-3′), precipitated, reverse-transcribed with 5 U of AMV RTAse (Promega) during 1 h at 38 °C, and PCR amplified in the presence of SA252 and SA253 oligonucleotides. The resulting DNA was then gel purified and submitted to another in vitro transcription. This cycle was repeated several times and DNA was cloned, into the *Kpn*I/*Pst*I sites of pBluescript II SK(+) (Stratagene) at various stages of selection-amplification. At least 20 individual clones were sequenced per protein. Note that the SELEX process using 9G8 recombinant protein has been done up to the eighth round in the presence or absence of 40 μM Zn^{++} without significant changes in the selected RNA population (data not shown), most likely because zinc remains tightly complexed with the recombinant 9G8 that was purified under nondenaturing conditions.

RNA–protein interactions

Plasmid DNAs corresponding to the RNA used for interaction studies were linearized at an *Xma*I site and transcribed in vitro with T7 RNA polymerase, under two different conditions (low or high [^{32}P]-labeling for gel shifts or UV crosslinking

assays, respectively). The gel-shift assays were performed in 25 μL-assays containing 10 mM HEPES, pH 7.9, 200 mM KCl, 20 mM NaCl, 0.1% Nonidet-P40, 10% glycerol, 1 mM DTT, 35 U RNAsin, and 0.8–1 μg BSA. The different proteins (75 ng for comparative analysis and less for the other experiments, see Figure and Table legends) and RNA (8,000 cpm, 5–10 fmol) were incubated in the binding buffer during 20 min at 20 °C in the presence of 250 ng of *E. coli* tRNA or in its absence (K_d determination). The resulting complexes were resolved on a 6% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide ratio of 40/1) in 0.5× TBE. The quantification of the binding efficiency was performed by Fuji Bio-Imager counting. To calculate the apparent K_d, the fraction of active protein has been determined for the fusion proteins by saturation binding assays in the presence of an RNA excess. This fraction comprised between 48 and 64% for the GST-9G8, SC35, and ASF/SF2.

UV crosslinking and immunoprecipitations

For UV crosslinking experiments, 2 μL of standard nuclear or S100 extracts (Cavaloc et al., 1994) were preincubated for 10 min at room temperature, in the presence of 0.75 mM ATP, 25 mM creatine phosphate, 1 mM $MgCl_2$, 50 mM KCl, and 250 ng of *E. coli* tRNA, in 10-μL volumes. Interaction between proteins and RNA (2×10^5 cpm of highly [^{32}P]-labeled transcript), was performed in final concentrations of 150 mM NaCl and 50 mM KCl, in a 20-μL volume, for 15 min at room temperature. The reaction mixtures were then exposed to UV light (254 nm) for 10 min at 4 °C, at a distance of 2 cm, and subsequently treated with RNase (250 ng of RNase A and 100 U of RNase T1), for 40 min at 37 °C. After addition of an equal volume of 2× loading buffer, the samples were analyzed by SDS-PAGE in a 12% gel, which was then dried and autoradiographed.

Immunoprecipitation assays were performed under the same conditions as above up to the RNase treatment stage, in volumes 1.5 larger, and only RNase T1 (200 U) was used. The samples were then diluted to a volume of 100 μL with IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% NP 40) and added to antibodies (monoclonal αSC35 and polyclonal α9G8 and αASF directed against the N (9G8) or C terminal (SC35 and ASF) 13–15 residue peptides) prebound to protein A or protein G-Sepharose. Each antibody recognizes exclusively its corresponding SR protein. After an overnight incubation at 4 °C, followed by three washing steps in a similar buffer containing 0.25% NP 40, proteins bound to the Sepharose were eluted with 2× loading buffer, for 5 min at 90 °C. The beads were eliminated by centrifugation and the samples were analyzed as described above.

Constructs and in vitro splicing

The pFib construct was derived from the pAdED plasmid (Lavigueur et al., 1993), which has been truncated by the deletion of a *Bsp*MI restriction fragment. The natural splicing enhancer (located between the *Bst*EII and *Stu*I sites) has been removed and replaced by a linker that recreates the natural *Bst*EII and *Stu*I restriction sites and contains also one *Kpn*I and one *Pst*I site for the insertion of the exonic splicing enhancer sequences. The sequences, tested for their splicing enhancer activity, originate from our SELEX experiments or from Tacke & Manley (1995). Annealed oligonucleotides containing the selected 20 mer sequences, flanked 5′ and 3′ by a 8-bp and a 6-bp invariant sequence, present in the original SELEX matrix, were inserted in the *Kpn*I and *Pst*I sites of the pFib plasmid. The pFGFR2 plasmid was derived from the pSAM-RK13 construct (Del Gatto & Breathnach, 1995), in which the natural splicing silencer was removed and replaced by sequences to be tested.

The Sp1 construct contains the *Xma*I-*Xba*I from the previously described E1A unit (Schmitt et al., 1987). We introduced four unique restriction sites, at positions +10 after the 3′ splice site (*Hpa*I), +35 (*Bam*HI), +60 (*Eag*I), and +79 (*Kpn*I). The exon 2 sequence was inverted between the *Hpa*I and *Xba*I sites and results in a novel construct (Sp1 Ex2 inv) that is not spliced in vitro (C.F. Bourgeois & J. Stévenin, unpubl.). The same selected sequences, as above, were inserted between the *Kpn*I (+42 after the 3′ splice site in the new construct) and *Bam*HI (+88) sites of the Sp1 Ex2 inv plasmid.

Standard in vitro transcription and splicing assays were performed as described previously (Schmitt et al., 1987; Cavaloc et al., 1994). The complementation experiments were performed in the presence of 8–9 μL of S100 cytoplasmic fraction and 3–4 μL of 20–40% ammonium sulfate nuclear fraction, in a total assay of 25 μL, as described (Tacke & Manley, 1995). These assays were supplemented with 400–800 ng of individual SR proteins. Recombinant 9G8, ASF/SF2, and SC35 were purified as described previously (Cavaloc et al., 1994; Gallego et al., 1997). Recombinant 9G8Zn^m was cloned in pVL1492 expression vector using the same strategy as for the 9G8Zn$^m\Delta$RS protein and immunopurified as the wild-type 9G8 protein (Cavaloc et al., 1994). Recombinant SRp20 was partially purified from baculovirus infected Sf9 cells as in Zahler et al. (1992), except that the protein is precipitated with 40% ammonium sulfate before $MgCl_2$ precipitation.

NOTE ADDED IN PROOF

T. Schaal and T. Maniatis (*Mol Cell Biol 19*, March issue) have isolated splicing enhancers by functional SELEX. The sequence of some of their enhancers, which are activated or recognized by 9G8 or SRp20, match very well the consensus that we have identified for these SR proteins.

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